ANNEX 5

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Comparison of four bifunctional reagents for coupling peptides to proteins and the effect of the three moieties on the immunogenicity of the conjugates

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Peptide-carrier conjugates are widely used to raise antipeptide antibodies. In a model system using angiotensin and tetanus toxoid as the peptide and the carrier protein respectively, four cross-linking reagents were employed to study their effect on the immunogenicity of the conjugates. Optimization of the conjugation method for these heterobifunctional reagents, all succinimidyl active esters, resulted in well-defined conjugates of predictable composition. ELISA assays were performed to compare the antigenicity and the immunogenicity of the conjugates. The antipeptide antibody titres were of the order of 2×10^4–2×10^5. The anti-carrier antibody titres were high, in spite of the modification of the protein. Three of the four coupling reagents used for conjugation were of the 'maleimide' type: succinimidyl 6-(*N*-maleimido)-*n*-hexanoate (MHS), succinimidyl 4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC) and succinimidyl *m*-maleimidobenzoate (MBS). One coupling reagent contained an activated disulphide: succinimidyl 3-(2-pyridyldithio)propionate (SPDP). The constrained linkers originating from SMCC and MBS induced very high *linker*-specific antibody levels. The more flexible non-aromatic linkers originating from MHS and SPDP showed almost no reactivity. For this reason and since the thioether linkage is more stable than the disulphide bond, we recommend MHS as the crosslinking reagent of choice.

Key words: Antipeptide antibody; Conjugation method; Cross-linking reagent; Linker-specific antibody; Peptide-protein conjugate

Introduction

Antipeptide antibodies have become important tools in many research fields; they have been used to identify new gene products, to analyse the functional domains of enzymes, for protein purification, for assaying proteins in immunochemical assays and to check the potential efficacy of synthetic peptide vaccines (for review see Moser et al., 1985; Walter, 1986).

In general, peptides consisting of 10–30 amino acid residues do not elicit antibodies following

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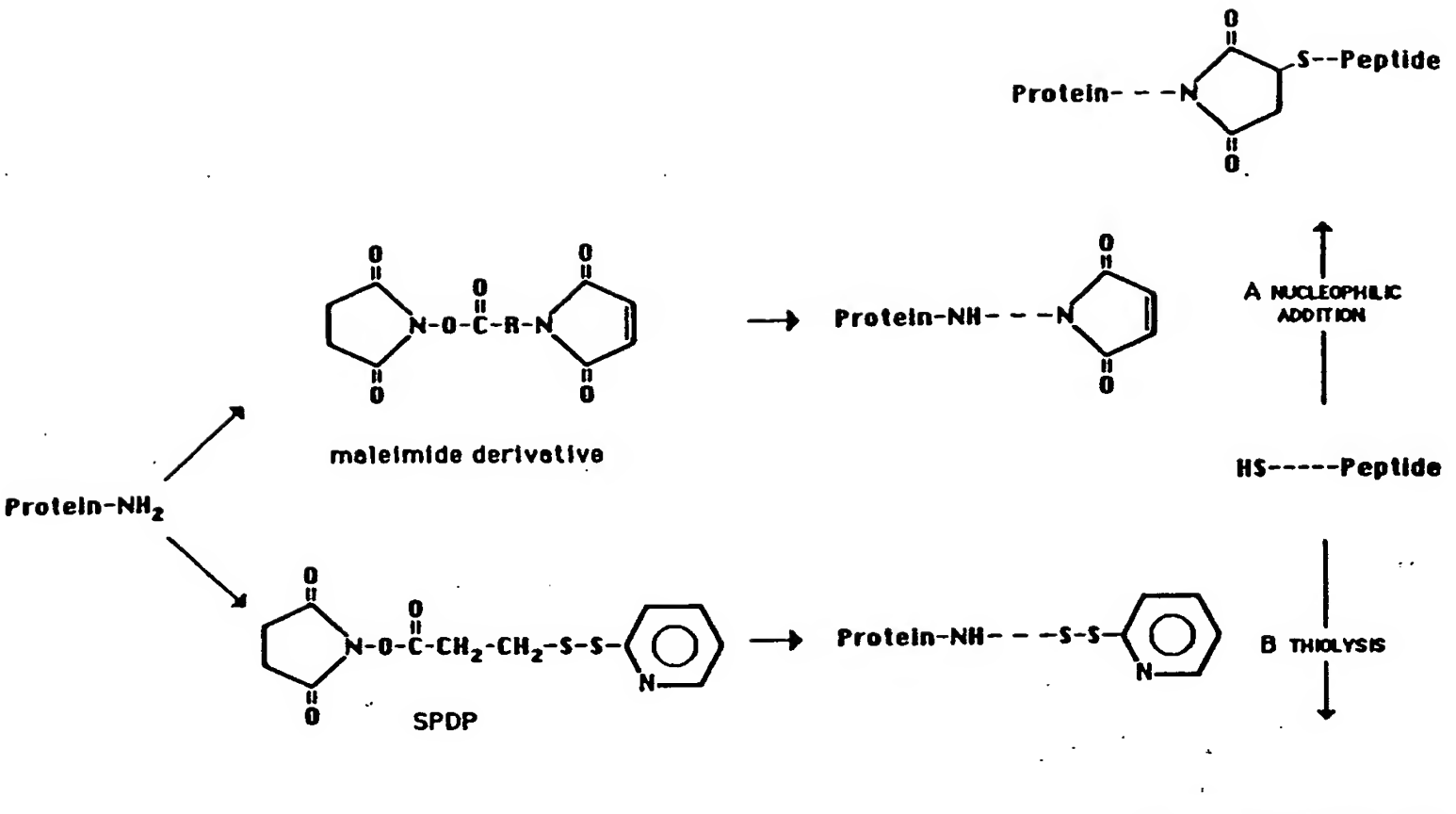
The *nomenclature* used is in accord with the rules and recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature: Eur. J. Biochem. 138, 9–37 (1984).

Abbreviations: Ata, acetylthioacetyl; MHS, succinimidyl 6-(*N*-maleimido)-*n*-hexanoate; Mh, 6-maleimidohexanoyl; SMCC, succinimidyl 4(*N*-maleimidomethyl)-cyclohexane-1-carboxylate; MBS, succinimidyl *m*-maleimidobenzoate; SATA, succinimidyl *S*-acetylthioacetate; SPDP, succinimidyl 3-(2-pyridyldithio)propionate; NHMe, methylamide; OPfp, pentafluorophenyloxy; TT, tetanus toxoid; DT, diphtheria toxoid; A1, [Val^5]-angiotensin I.



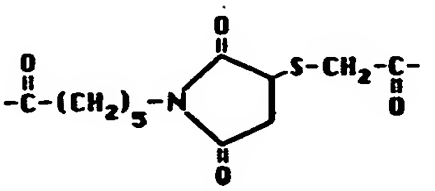
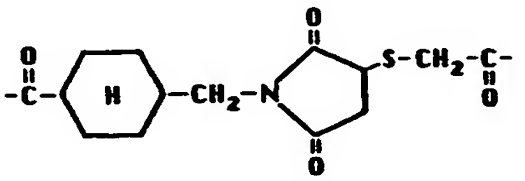
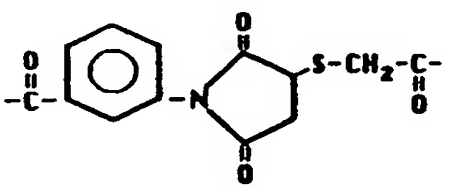
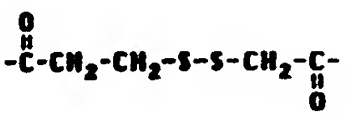
Conjugation method	Cross-linking reagent	carrier---SPACER---peptide
route A	MHS	
route A	SMCC	
route A	MBS	
route B	SPDP	

Fig. 1. Synthesis of protein-peptide conjugates.

immunization. To induce immunogenicity, peptides are coupled to macromolecular carriers, usually proteins such as bovine serum albumin (BSA), keyhole limpet haemocyanin (KLH) and ovalbumin, to synthetic carriers such as multichain poly(DL-Ala)-(L-Lys) (Audibert, 1982) and polytuftsin (Trudelle, 1987) or they are incorporated into liposomes, micelles or immuno-stimulating complexes known as iscoms (Morein et al., 1984). In general, bifunctional reagents are required to couple peptides to proteins. Since peptides and proteins contain several functional groups, conjugation using carbodiimides (Goodfriend et al., 1964; Davis et al., 1984) or homobifunctional reagents (e.g., glutardialdehyde (Avrameas, 1969; Pfaff et al., 1982) and bis-diazotized benzidine (Walter et al., 1980; Tamura and Bauer, 1982)) generates a great number of different products ('chaos' coupling). In order to obtain the best-defined product heterobifunctional cross-linkers should be used in such a way that the peptide will be coupled specifically and in a predictable fashion to the carrier. Various aspects of chemical cross-linkers and the modification of proteins have been reviewed by Han et al. (1984) and Feeney (1987).

A useful method for preparing peptide-carrier conjugates involves taking advantage of the fast nucleophilic addition of a thiol group to the double bond of a maleimide (Marrian, 1949). The amino groups of the carrier can be modified to an adjustable extent with an active ester (e.g., a succinimidyl ester) bearing a maleimide moiety. Subsequently the maleimido groups are allowed to react with peptides bearing a sulphydryl group (Fig. 1, route A).

Peptides devoid of sulphydryl groups (i.e., containing no cysteinyl residues) can also be thiolated in a reproducible way by functionalization of one particular amino group. Homocysteine thiolactone (Lee et al., 1980) and the succinimidyl esters of *S*-acetylthioacetic acid (SATA) (Duncan et al., 1983) and 3-(2-pyridyldithio)propionic acid (SPDP) (Carlsson et al., 1978) are examples of reagents which have been used for this purpose. Since both the peptide and the protein in the latter route are unified through acylation of amino groups it is evident but nonetheless important to note that thiolation and maleylation of carrier and peptide can be reversed. A further method of controlled conjugation involves the generation of S–S (sulphanyl or disulphide) links by the thiolysis of activated disulphides originating from the acylation of a protein or a peptide with SPDP (Fig. 1, route B).

We have investigated the effect of several coupling reagents on the antigenicity and the immunogenicity of the conjugates, taking into account the number of conjugated peptide molecules and also ensuring specific conjugation. The model peptide used was the decapeptide [Val^5]-angiotensine I and the carrier was tetanus toxoid. Three maleimide derivatives have been used to modify the carrier protein: succinimidyl *m*-maleimidobenzoate (MBS) (Kitagawa and Aikawa, 1976); succinimidyl 4-(*N*-maleimidomethyl)-*cyclo*-hexane-1-carboxylate (SMCC) (Yoshitake et al., 1979); succinimidyl 6-(*N*-maleimido)-*n*-hexanoate (MHS) (Keller and Rudinger, 1975; cf. Wünsch et al., 1985). For comparison a disulphide linked conjugate was synthesized using SPDP to modify the carrier.

Materials and methods

Angiotensin and ornithine derivatives

[Val^5]-angiotensin I (AI) was available from our organic chemistry laboratory. The decapeptide was acylated with Fmoc-Orn(Boc)-OPfp, deprotected by β-elimination of the Fmoc group using a base and further functionalized with an acetylthioacetyl group to give Ata-Orn-AI or with the maleimide derivative MHS resulting in Mh-Orn-AI. The mono-hydrochlorides of Ata-Orn-NHMe and Mh-Orn-NHMe were prepared following standard procedures.

Bifunctional reagents

Succinimidyl *m*-maleimidobenzoate (MBS) was obtained from Pierce (Rockford, U.S.A.). Succinimidyl 6-(*N*-maleimido)-*n*-hexanoate (MHS) was a gift from Boehringer (Mannheim, F.R.G.). Succinimidyl 4-(*N*-maleimidomethyl)-*cyclo*-hexane-1-carboxylate (SMCC) was synthesized with modifications according to the methods of Yoshitake et al. (1979) and Wünsch et al. (1985). Succinimidyl *S*-acetylthioacetate (SATA) was synthe-

sized according to Duncan et al. (1983). Succinimidyl 3-(2-pyridyldithio)propionate (SPDP) was obtained from Pierce (U.S.A.).

Carriers

Tetanus toxoid (TT) and diphtheria toxoid (DT) were obtained from Dr. J. Nagel (RIVM). Tetanus toxoid contained 48 equivalent amino groups (per 150 kDa molecule) available for coupling and for diphtheria toxoid this ratio was 9 per 62 kDa. In both cases these values were determined by the 2,4-dinitrophenyl method of Sanger (reviewed by Needleman, 1970).

Conjugation method

(a) Derivatization of the carrier. The coupling method was a modification of the procedures of Lee et al. (1980) and of Green et al. (1982): 1 ml of carrier solution containing 10 mg/ml of protein was equilibrated in phosphate-buffered saline (0.1 M phosphate, pH 8.0–8.5; 0.9% NaCl) by gel filtration on PD-10 Sephadex (Pharmacia, Sweden). A bifunctional reagent (MHS, SMCC, MBS or SPDP) was added as a 1% solution in DMF at a molar ratio corresponding to the desired number of peptides to be conjugated, i.e., 1.5 equivalents of succinimidyl ester per amino group to be coupled. Following a reaction period of 5 min the reaction mixture was subjected to gel filtration using PD-10 Sephadex equilibrated in phosphate buffer (0.1 M, pH 6.66, 0.9% NaCl, 5 mM EDTA). The amount of maleimido groups coupled was determined using 5,5′-dithiobis(2-nitrobenzoic acid), DTNB, Merck (Sedlack and Lindsay, 1968). Prior to this thiol group detection the maleimido conjugates were treated with excess β-mercaptoethanol. The amount of SPDP coupled was determined by measuring the release of thiopyridone following reduction of the disulphide bond (Carlsson et al., 1978) with DTE (1,4-dithioerythritol, Fluka, Switzerland). The activated carriers were frozen and stored at −20 °C.

(b) Deprotection of the peptide. Two methods were used for deacetylation of Ata-Orn-angiotensin. The in situ method of Duncan et al. (1983) with hydroxylamine hydrochloride in a phosphate buffer (pH 6.5, 0.1 M). A faster method permitting the easy isolation of the *N*-thioacetylpeptide was performed with a hard base as used in the 'short-high' deprotection method of Msc compounds (Tesser and Balvert-Geers, 1975; Boon and Tesser, 1985). Although this procedure includes a neutralization step after about 5 s, it cannot be used in conjunction with maleyl groups and some disulphides.

(c) Coupling. Immediately after deprotection of the angiotensin derivative the peptide was added to the modified carrier in a 2:1 molar ratio. The reaction mixture was stirred for 1 h at room temperature. For TT modified with SPDP, the coupling was monitored by thiopyridone release with reaction times up to 24 h. The conjugated product was dialysed against PBS (0.01 M phosphate, 0.9% sodium chloride; pH 7.3).

(d) Analysis of the conjugates. All protein determinations were performed using the method of Peterson (1977). Spectrophotometric measurements were made with a Cary 118 instrument at ambient temperature. Amino acid analysis was used to determine the amount of peptide in the conjugate. Peptides were hydrolysed in sealed evacuated vials in 5.7 N hydrochloric acid (Merck, suprapur, Darmstadt, F.R.G.) at 110 °C for 24 h, carriers and conjugates under the same conditions for 48 h. Hydrolysates were analysed on a Varian LC 5000 analyser. HPLC-gel filtration on a TSK-4000 column (eluent 0.01 M phosphate; 0.1 M sodium chloride; pH 6.8; flow rate 1 ml/min) was used to analyse the peptide conjugates bearing different amounts of peptide. The antigenicity of the conjugates was tested in an ELISA. An angiotensin-TT conjugate coupled with glutardialdehyde (supplied by Vishna Kanhai, RIVM, Bilthoven) was used as a reference.

Enzyme-linked immunosorbent assay (ELISA)

ELISAs were performed in PVC microtitre plates (Flow Laboratories, Scotland). PBS with 0.01% Tween 20 (PBST) was used as the washing buffer and PBST containing 0.5% bovine serum albumin was used as the diluent for the samples and the antisera. The substrate was prepared by adding 1.67 ml of a solution of 3,3′,5,5′-tetramethylbenzidine (Pierce, U.S.A.) in dimethylsulphoxide to 100 ml citrate buffer pH 5.5. Just before use 10 μl of 30% (w/v) hydrogen peroxide was added. The antigenic activity of the conjugates was tested

in an ELISA. Microtitre plates were coated with 100 μl anti-TT sheep antiserum (SATS) per well. Following overnight incubation at room temperature, the plates were washed twice. Subsequently, 100 μl of a solution containing conjugate or TT were added and the plates were incubated at 37 °C for 90 min. After washing as before rabbit anti-angiotensin antiserum (Calbiochem-Behring, La Jolla, U.S.A.) or a blank serum was added and the plates incubated for 90 min at 37 °C before a further wash stage. Finally, the plates were incubated with peroxidase-labelled sheep anti-rabbit IgG or with enzyme-conjugated sheep anti-TT serum (100 μl, 1 h, 37 °C) and washed three times. 100 μl of substrate were added and the reaction stopped by the addition of 2 M sulphuric acid (100 μl). The optical density at 450 nm was measured in a Titertek-Multiskan spectrophotometer (Flow Laboratories, Scotland).

TABLE I

ACTIVATION OF THE LYSYL GROUPS IN TETANUS TOXOID

Coupling reagent	pH	Time (min)	Ratio linker/ lysyl added	Coupling ratio	Yield [a] (%)
MHS	6.7	60	3.6	0.35	10
	6.7	60	1.8	0.22	12
	6.7	60	0.9	0.13	14
	6.7	60	0.45	0.06	14
	6.7	60	0.23	– [b]	–
	8.0	10	0.63	0.35	56
	8.5	5	0.94	0.50	53
	8.5	5	0.94	0.48	51
	8.5	5	0.52	0.38	72
	8.5	5	0.38	0.27	71
MBS	8.0	10	0.63	0.35	56
SMCC	8.0	10	0.63	0.29	46
	8.5	5	0.20	0.14	68
SPDP	8.0	10	0.63	0.31	49
	8.5	5	0.5	0.39	77
	8.5	5	0.5	0.34	69
	8.5	5	0.5	0.36	73
	8.5	5	0	0	0
	8.5	5	0.11	0.07	61
	8.5	5	0.33	0.24	71
	8.5	15	0.33	0.26	78
	8.5	5	0.90	0.62	69

[a] Yield: amount of reagent coupled compared to the amount added.
[b] Not determined, value below detection limit.

TABLE II

DETERMINATION OF THE PEPTIDE/CARRIER RATIO OF CONJUGATES

Conjugate code	SPDP added [a]	SPDP calc. [b]	SPDP determ. [c]	Amino acid	
				Mean [d]	Orn [e]
BC1	24.0	16.8	18.5	19.1	21.0
BC2	18.0	12.6	10.7	8.5	9.0
BC3	9.0	6.3	5.9	4.2	6.3
BC4	4.5	3.2	4.2	4.0	4.7
BC5	3.4	2.4	2.5	4.2	4.2

[a] Equivalents of SPDP added are corrected values, based on the succinimidyl contents.
[b] Calculated number of SPDP coupled, assuming the coupling efficiency was 70%.
[c] Number of SPDP coupled, determined by thiopyridone release following reduction with DTE.
[d] Amount of peptide coupled, determined by amino acid analysis, taking the mean of the difference between TT and conjugate (Briand et al., 1985).
[e] Amount of peptide coupled, determined by amino acid analysis, with reference to the ornithine content of the introduced peptide.

Immunization

6-week-old NIH male mice were immunized intraperitoneally with 10 μg of the conjugate, emulsified in complete Freund's adjuvant. After 6 weeks the mice were boosted with antigen in incomplete Freund's adjuvant and then bled 2 weeks later.

Immunogenicity

The antibody responses to angiotensin, spacer and carrier were determined using the ELISA. For angiotensin antibody responses, microtitre plates were precoated with 0.1% glutardialdehyde in PBS (pH 7.2, 150 μl/well) for 2 h at room temperature and washed twice. Angiotensin, its derivative, the diphtheria conjugates (see Table III) and TT were incubated overnight at room temperature (1 μg/well). The antigen solution was discarded and the plates were saturated with BSA (1% in PBST) for 1 h at room temperature. The plates were incubated with serial dilutions of antiserum. The assay was completed as described previously, using peroxidase-labelled sheep anti-mouse IgG conjugate at a 1/500 dilution.

Results

Activation of the carrier

The reaction efficiencies of the succinimidyl esters MHS, SMCC, MBS and SPDP with the available lysyl side chains in tetanus toxoid were tested by the addition of different amounts of the reagents. At pH 6.7 and using a 1 h reaction time the coupling efficiency (the fraction of the reagent molecules reacting) amounted to just 10%. Increasing the pH and lowering the reaction time permitted activation of a carrier in a predictable manner, i.e., at pH 8.5 the reactive groups were introduced with an efficiency of about 70 ± 4% within 5 min (c.f. Table I).

Loading density versus antigenicity and immunogenicity

In a preliminary experiment tetanus toxoid was thiolated with different amounts of SPDP, i.e., 0, 16, 32 and 48 mol/molTT and subsequently conjugated with Mh-Orn-angiotensin. During dialysis of the conjugates a precipitate was formed. HPLC-gel filtration of the soluble fraction confirmed that a higher peptide density leads to less soluble conjugates (Fig. 2). Conjugate I (Fig. 2*A*) modified with coupling reagent but containing no peptide showed no changes in molecular size in the main component compared to unmodified tetanus toxoid (Fig. 2*E*). From Fig. 2*B* it could be concluded that conjugate II, bearing 16 mol peptide/mol TT, contained components with molecular weights which differed from both modified (Fig. 2*A*) and unmodified (Fig. 2*E*) tetanus toxoid. The heterogeneity in the molecular weight of the tetanus toxoid, was probably related to the formaldehyde treatment used during toxoid preparation (Bizzini et al., 1970). A reference conjugate, i.e., tetanus toxoid and angiotensin coupled with glutardialdehyde, showed a similar (although less pronounced) elution profile (Fig. 2*F*). The dialysates III and IV with loading densities of 32 and 48 contained (almost) no soluble conjugate fraction. The peaks emerging with retention times of 27–28 min arose from the modification of tetanus toxoid by the coupling reagent (see Figs. 2*A* and 2*E*). The exact composition of these components was not clear although the elution profiles measured at 280 nm suggested an aromatic component (results not shown).

Testing the antigenicity of the conjugates in an ELISA using the indirect coating procedure, we found that the response diminished in spite of the increasing amount of incorporated angiotensin (Fig. 3). This phenomenon is probably an effect of the ELISA protocol: the indirect coating of the conjugate is less efficient with higher loading densities.

TABLE III

SPECIFICATION OF THE LINKER CONJUGATES USED FOR IMMUNIZATION AND DETECTION IN ELISA

Conjugate code	Hapten	Linker	Carrier	Peptide/carrier ratio		
				Reagent [a]	Orn [b]	AA [c]
C1	Ata-Orn-NHMe	MHS	TT	17	17	
C2	Ata-Orn-NHMe	SMCC	TT	14	20	
C3	Ata-Orn-NHMe	MBS	TT	17	12	
C4	Ata-Orn-NHMe	SPDP	TT	15	19	
C5	Ata-Orn-AI	MHS	TT	16	19	11
C6	Ata-Orn-AI	SMCC	TT	13	16	12
C7	Ata-Orn-AI	MBS	TT	17	16	15
C8	Ata-Orn-AI	SPDP	TT	13	13	10
C9	Ata-Orn-NHMe	MHS	DT	7	6	
C10	Ata-Orn-NHMe	SMCC	DT	3	5	
C11	Ata-Orn-NHMe	MBS	DT	7	4	
C12	Ata-Orn-NHMe	SPDP	DT	5	8	

[a] Amount of peptide coupled determined by measurement of the incorporation of the functional group.
[b] Amount of peptide coupled determined by amino acid analysis, based on the number of ornithyl residues.
[c] Amount of peptide coupled determined by amino acid analysis according to the method of Briand et al. (1985).

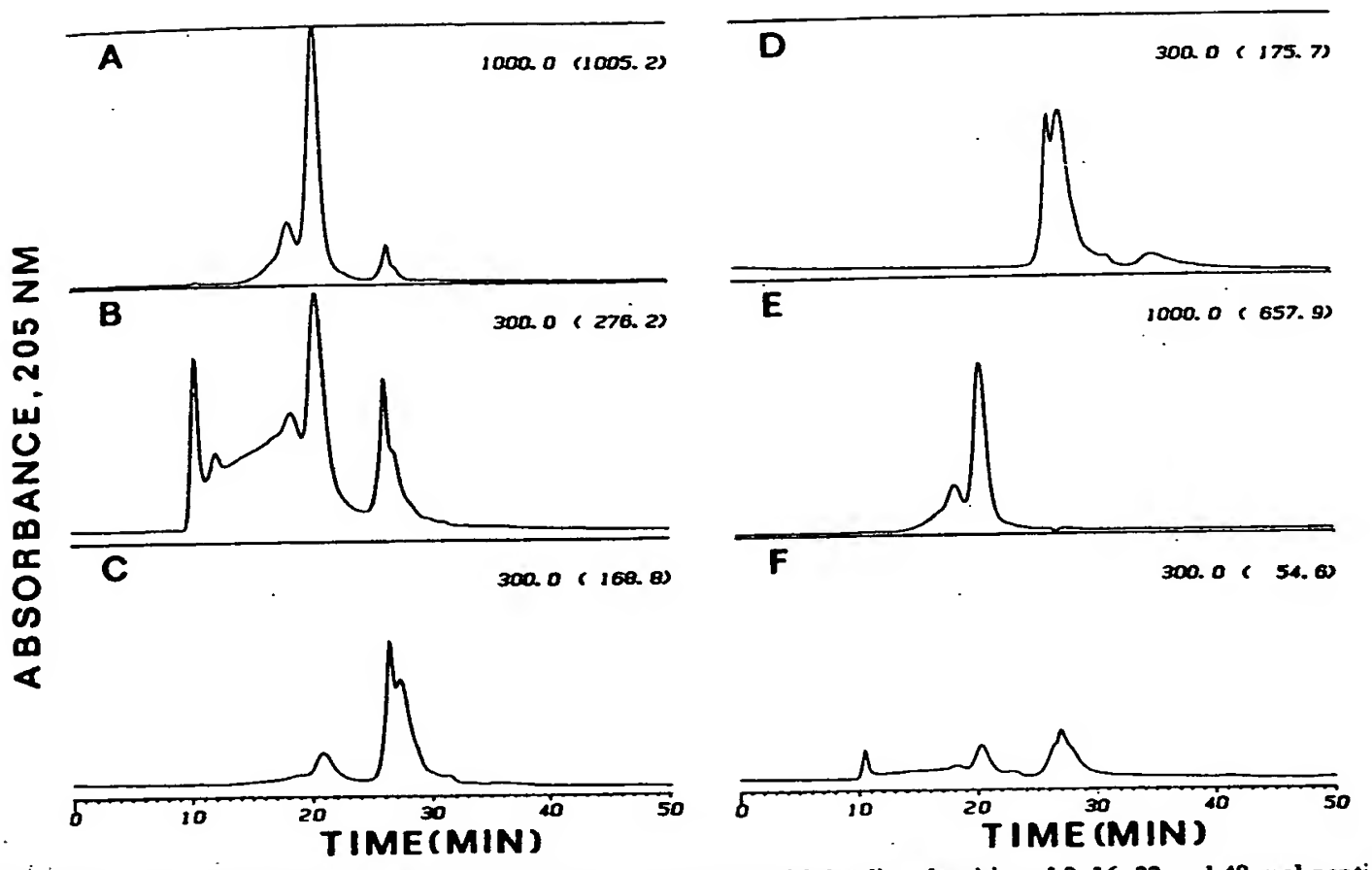


Fig. 2. HPLC gel filtration-elution profiles for angiotensin-TT conjugates with loading densities of 0, 16, 32 and 48 mol peptide/mol TT (Figs. 2*A*–2*D* resp.) compared to TT (Fig. 2*E*) and an AI-TT conjugate coupled with glutardialdehyde (Fig. 2*F*). Figs. 2*A* and 2*E* were recorded at an attenuation of 1000 mAU; Figs. 2*B*, 2*C*, 2*D* and 2*F* at an attenuation of 300 mAU. The absorbances of the peak maxima are indicated in brackets.

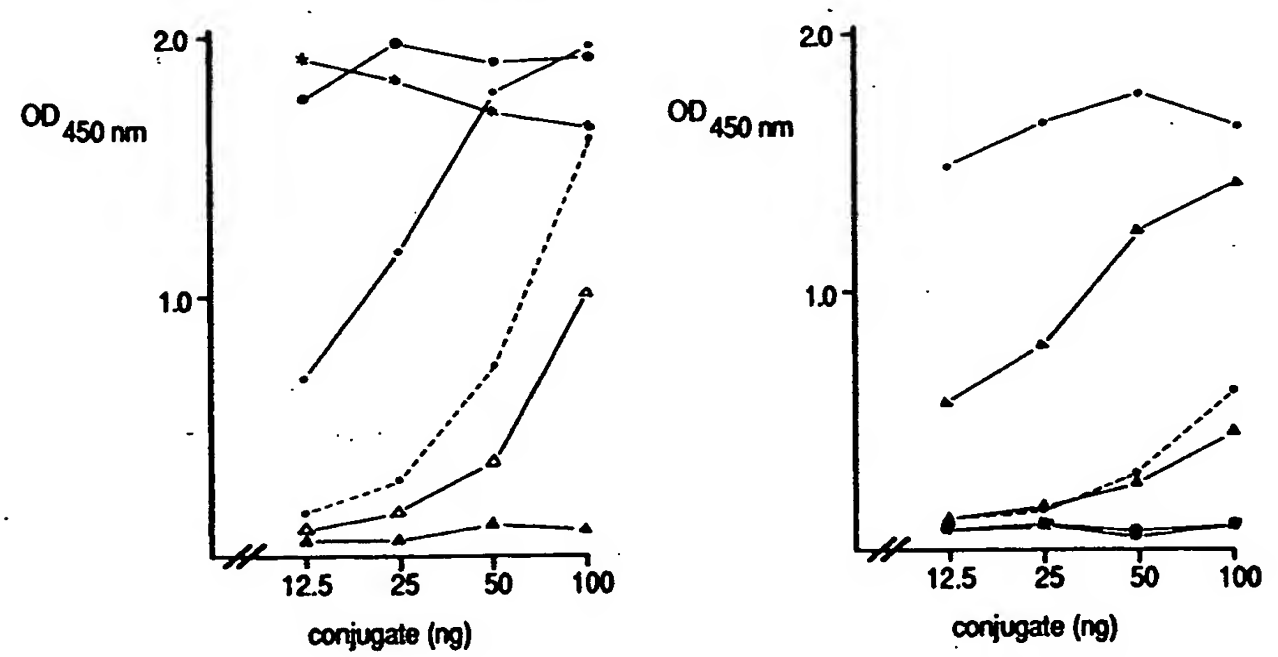


Fig. 3. Antigenicity of AI-TT conjugates with various loading densities: 0 (•–•–•); 16 (.–.–.); 32 (Δ–Δ–Δ); 48 (▲–▲–▲) mol AI/mol TT, compared to TT (*–*–*) and an AI-TT conjugate coupled with glutardialdehyde (.—.—.), tested in an ELISA with anti-TT (*A*) and anti-angiotensin (*B*) antisera. The amount of conjugate given is the amount added per well. Preparation of the antigen: 10 μg conjugate or TT was dissolved in 8 M urea. After 1 h the solution was diluted to 10 ml with water. A serial dilution of antigen (100 μl) in buffer was added to the wells and the ELISA was performed as described in the materials and methods section.

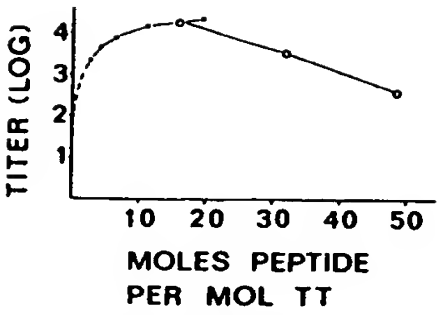


Fig. 4. The immunogenicity of angiotensin-TT conjugates with various loading densities. ELISA titres from two experiments are shown, one in the 'non-soluble' conjugate range (○———○), the other in the 'soluble' conjugate range (■———■) (see text). Titre is expressed as the reciprocal of the antiserum dilution which resulted in 50% of maximal absorption. Blood samples were taken 56 days after the first immunization. A pool of sera from eight mice were examined for each conjugate. Titres were determined in duplicate.

Taking the non-loaded conjugate as a reference, the immunogenicity of the conjugates, as measured by antipeptide antibody production, decreased with *in*creasing antigen density (Fig. 4). This was shown to be due to decreasing solubility in an experiment in which loading ratios in the 'soluble conjugate' range (comprising 2.5, 4, 6, 11 and 19 peptide units per 150 kDa of carrier (Table II)) gave rise to high antibody titres (Fig. 4). The titre of the glutardialdehyde conjugate (see materials and methods section) was comparable with the titre of the conjugate with the highest density (48 mol peptide/mol TT): 2.4 in log units.

Specificity and cross-reactivity

Four linkers were compared for their effect on the immunogenicity of the conjugate, namely three maleimide derivatives and one activated disulphide. The reagents differ through the nature of the links exerted (Fig. 1). It is supposed that there would be a negligible contribution to the diversity of the antibodies from the small structural difference in the link between peptide and carrier arising from the reversal of maleylation and thiolation of peptide and carrier.

The effect of the spacer on the immunogenicity of the resulting conjugate was investigated in an ELISA by coating the microtitre plates with conjugates consisting of an unrelated carrier protein (i.e., diphtheria toxoid), and a 'false' peptide (ornithine methylamide, Orn-NHMe). Ornithine methylamide was functionalized with SATA to give Ata-Orn-NHMe. Immunization was performed with Ata-Orn-angiotensin coupled to tetanus toxoid, and with the corresponding conjugate of Ata-Orn-NHMe (Table III). In Table IV the immunogenicity of the conjugates is illustrated. All four angiotensin conjugates elicited antibodies against the peptide and the peptide derivative to a large extent. Anti-tetanus toxoid

TABLE IV

TITRES REPRESENTING IMMUNOGENICITY OF ANGIOTENSIN-TETANUS TOXOID CONJUGATES COMPARED TO METHYLAMIDE-TETANUS TOXOID CONJUGATES

Titre was defined as the reciprocal of the dilution of antiserum which resulted in 50% of maximal absorption. The composition of the conjugates is specified in Table III.

Immunogen	Anti-X, X =						
Tetanus Toxoid conjugates (linker)	Peptide derivatives		Diphtheria conjugates (linker)				Carrier
	AI	AtaOrnAI	C9 (MHS)	C10 (SMCC)	C11 (MBS)	C12 (SPDP)	TT
C1 (MHS)	550	1000	$>10^5$	$>10^5$	$>10^5$	$>10^5$	$>10^6$
C2 (SMCC)	850	1000	$>10^5$	$>10^5$	$>10^5$	$>10^5$	$>10^6$
C3 (MBS)	750	900	40000	36000	$>10^5$	55000	$>10^6$
C4 (SPDP)	630	670	270	320	320	700	$>10^6$
C5 (MHS)	140000	160000	3400	280	210	225	$>10^6$
C6 (SMCC)	96000	100000	9700	54000	1800	245	$>10^6$
C7 (MBS)	155000	200000	550	660	$>10^5$	260	$>10^6$
C8 (SPDP)	16500	40000	150	180	320	370	$>10^6$

titres were high ($> 10^6$) despite modification of the tetanus toxoid carrier. In the case of all conjugates except the SPDP-derived C4 and C8 conjugates there was a high antibody response to the homologous linker conjugate. However, cross-reactions occurred, especially with the anti-blank conjugate antibodies elicited by C1 (MHS), C2 (SMCC) and C3 (MBS). The anti-angiotensin conjugate antibodies showed cross-reactions to a much lesser extent and there were few cross-reacting antibodies elicited by C5 (MHS) and C8 (SPDP).

Discussion

Reaction conditions

Lee et al. (1980) reported that MHS reacted with TT with a high efficiency until 15 maleimido groups per 10^5 Da had been introduced. The reaction ran at pH 6.7 and required 60 min but for higher densities an excess of MHS had to be added. In order to increase the reaction rate, the pH of the reaction mixture should be increased: Boon (1985) found that the reaction of 2-(methylsulphonyl)ethyl succinimidyl carbonate (Msc-ONSu) with the ϵ-amino groups of the (19) lysyl residues in cytochrome *c* was completed within 5 min at pH 8.5 and showed that the reaction proceeded stoichiometrically. Hydrolysis of the active ester was relatively slow (see also Aldwin and Nitecki (1987) and Anjaneyulu and Staros (1987)). In the present work it has been demonstrated that if the reaction is performed at a higher pH, it is possible to obtain a high density of maleimido groups without the requirement for a large excess of MHS. However, the 'spontaneous deterioration' of the succinimidyl ester moieties in the reagents of Fig. 1 should be taken into account and their actual activity should be determined beforehand.

The maleimido group of MHS proved to be 99% stable for at least 15 min at pH 8.5, confirming the observation of Wünsch et al. (1985). The maleimido group in MBS is less stable and therefore the activation was performed at pH 8.0 with a molar ratio of MBS/amino group of 2. Subsequent gel filtration of the reaction mixture should be carried out at pH 6.7. Lee et al. (1980) have reported that the reaction proceeds in the absence of thiol groups with an efficiency of only 10% over 30 min and therefore the possibility of nucleophilic addition of amino groups can be discounted. Storage of the modified carrier at $-20\,^{\circ}C$ is recommended to slow down any functional deterioration catalyzed by imidazolyl functions occurring in the protein (His). At $-20\,^{\circ}C$ the activated carrier is stable for at least a few months. At $4\,^{\circ}C$ the thiol binding activity decreases with a halftime of about 1 month.

Loading density

The concentration of cross-linking reagent during activation of the carrier determines the loading of the carrier only to a minor degree. Thus at a given pH the loading density appears only to depend on time. This effect is to be expected since the pH determines the number of reactive amino groups (p*Ka* ϵ-amino group = 10.5); this quantity is far outnumbered by the amount of succinimidyl ester molecules so the reaction appears to run at a given pH with near zero-order kinetics (Table I).

Determination of carrier load from amino acid analysis (cf. Briand et al., 1985) was found to be less accurate than from the number of maleimido groups introduced per molecule of carrier (cf. Lee et al., 1980). The latter method was particularly suitable when large peptides were to be coupled to a protein-carrier and the amino acid composition resembled that of the carrier. The intentional introduction of a diagnostic amino acyl group (Orn and Nle) was also another efficient method. Carrier modification is a stoichiometric reaction: reduction of the reaction time to 5 min at pH 8.5 diminishes the efficiency to about 70%.

Experiments with angiotensin I as a model peptide and tetanus toxoid as the carrier protein showed that an optimum loading density exists which amounts to 10–20 peptides/mol tetanus toxoid. This number is somewhat lower than that reported by Stevens et al. (1981) ($25/10^5$ kDa tetanus toxoid) but the curve is truncated by the insolubility of the higher substituted carrier molecules (in our case at a molar ratio of 32 and higher). Stevens et al. apparently had no solubility problems. Masking of determinants could be an explanation for decreasing immunogenicity at increasing densities. If the anti-TT response is measured following urea denaturation (to effect solu-

bility), the TT-response decreases with increasing density, whereas the angiotensin response decreases only slightly. Masking of carrier determinants could be the explanation of this phenomenon but masking of hapten determinants is also possible since closely packed angiotensin residues would impair recognition by the immune system.

Specificity and cross-reactivity

The effect of several bifunctional reagents on the immunogenicity of the conjugate was investigated. MHS, MBS, SMCC and SPDP were used as cross-linkers in peptide-protein conjugation with angiotensin I as the peptide and tetanus toxoid as the carrier protein. The results showed that the immunogenicity of a glutardialdehyde conjugate was comparable to that of a highly substituted sulphur-linked conjugate. Since an excess of glutardialdehyde was routinely used, a reference conjugate was not included when the low density sulphur-linked conjugates were investigated.

The polyclonal antisera raised against the angiotensin conjugates included antibodies which reacted specifically with the unmodified decapeptide angiotensin I in an ELISA. There was no significant difference in reactivity with an angiotensin coat or with derivatized angiotensin I and titres ranged from 2×10^4 to 2×10^5.

The reactivity of the antisera towards tetanus toxoid was also very high, of the order of 10^6, irrespective of the coupling of the peptide. This suggests that the character of this carrier, chosen since it can be used for human immunization, was not changed profoundly.

Four [angiotensin]-linker-[tetanus toxoid] conjugates were used to raise antibodies. The antisera also contained antibodies directed against the linkers, since they cross-reacted with their homologous [ornithine methylamide]-linker-[diphtheria toxoid] conjugates. There were however, qualitative differences: (1) the SPDP spacer containing an aliphatic disulphide, showed almost no reactivity; (2) for the MHS linker, which results in a flexible aliphatic chain connected through a disulphide bridge to a succinimide ring, the antibody level was somewhat higher; (3) the rigid linkers produced by SMCC and MBS, containing an additional cycloaliphatic or aromatic ring, induced very high antibody titres, within the range 10^4–10^5.

It has previously been shown that cross-linkers such as glutardialdehyde, carbodiimide or reagents containing an aromatic moiety elicit antibodies directed against the spacer (Palfreyman et al., 1984; Briand et al., 1985; Bernatowicz and Matsueda, 1985). Aldwin and Nitecki (1987) demonstrated by Western blot analysis that peptide-protein conjugates cross-linked with a water-soluble active ester of 6-(*N*-maleimido) *n*-hexanoic acid did not induce detectable antibody specific for the spacer. The spacer originating from MHS used in the present study was the same as that investigated by Aldwin and Nitecki and our results confirm their findings quantitatively. The cross-linking agents MHS and SPDP are preferable to SMCC and MBS in terms of their lower potential for immunogenicity, greater flexibility and greater stability in aqueous solutions. A drawback of SPDP coupling is the resulting disulphide linkage, which confers a susceptibility to reductive cleavage by ubiquitously occurring thiol compounds. The thio-ether linkage resulting from the application of MHS is very stable (at least for 6 months at pH 6 at 4°C) (Yoshitake et al., 1982) and we conclude that MHS is the bifunctional reagent of choice for coupling peptides to proteins.

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